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# *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin

Keat H. Teoh, Devin R. Polichuk, Darwin W. Reed, Goska Nowak, Patrick S. Covello*

*Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Sask., Canada S7N OW9*

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**Abstract** **Artemisinin, a sesquiterpene lactone endoperoxide derived from the plant *Artemisia annua*, forms the basis of the most important treatments of malaria in use today. In an effort to elucidate the biosynthesis of artemisinin, an expressed sequence tag approach to identifying the relevant biosynthetic genes was undertaken using isolated glandular trichomes as a source of mRNA. A cDNA clone encoding a cytochrome P450 designated CYP71AV1 was characterized by expression in *Saccharomyces cerevisiae* and shown to catalyze the oxidation of the proposed biosynthetic intermediates amorpha-4,11-diene, artemisinic alcohol and artemisinic aldehyde. The identification of the CYP71AV1 gene should allow for the engineering of semi-synthetic production of artemisinin in appropriate plant or microbial hosts.**


*Keywords:* Artemisinin; Sesquiterpene; Cytochrome P450; Trichome; *Artemisia annua*

## 1. Introduction

A little over thirty years ago, as part of a search for new antimalarial drugs, Chinese scientists isolated a sesquiterpene lactone endoperoxide called qinghaosu or artemisinin (see Fig. 1) from *Artemisia annua* [1]. Artemisinin and its derivatives have since become the most important agents in the treatment of malaria, particularly in the form of artemisinin-based combination therapies (ACTs) [2].

As a member of the tribe Anthemideae in the Asteraceae, *A. annua* characteristically produces an essential oil rich in mono- and sesqui-terpenes which is sequestered in 10-celled biseriate glandular trichomes [3,4]. The overall composition of *A. annua* essential oil is dominated by monoterpenes including artemisia ketone, camphor, α-pinene and pinocarvone [4]. The sesquiterpene lactone artemisinin accumulates to levels of 0.01–1% of dry weight [5]. Recently, a reasonably clear picture of artemisinin biosynthesis has emerged as illustrated in Fig. 1 [6]. The identity of amorpha-4,11-diene as a biosynthetic intermediate was established, based on the presence of traces of amorpha-4,11-diene in *A. annua* extracts and the cloning and expression of cDNAs representing amorpha-4,11-diene synthase, a sesquiterpene cyclase [7,8]. Dihydroartemisinic acid is thought to be an intermediate produced by enzymatic oxidation at the C12 to carboxylate and reduction of the double bond at C11–C13 of amorpha-4,11-diene. The biochemical evidence reported recently [6] is consistent with cytochrome P450 (CYP) involvement for the initial hydroxylation of amorpha-4,11-diene and with distinct (soluble) enzyme activity required for subsequent oxidation to carboxylic acid.

In an effort to improve our understanding of the biosynthesis of artemisinin, and of sesquiterpene lactones in general, we undertook the molecular cloning of the enzyme involved in amorpha-4,11-diene oxidation. The expressed sequence tags (ESTs) generated from isolated trichomes of *A. annua* were used as a starting point for the molecular cloning and characterization of CYP71AV1, a multifunctional sesquiterpene oxidase.

## 2. Materials and methods

### 2.1. Chemicals and chemical analysis

(−)-α-Gurjunene, (+)-γ-gurjunene, (−)-alloisolongifolene, (+)-ledene and (+)-valencene were obtained from Sigma–Aldrich. Caryophyllene, limonene, α-pinene, β-pinene, pinocarveol and (+)-β-selinene were obtained from the Plant Biotechnology Institute terpene collection. (+)-β-Selinene (from celery seed) was confirmed by $^{1}$H NMR and EI$^{+}$-GC/MS and was found to contain a 4:1 mixture of β- and α-selinene.

Artemisinic acid was isolated from *A. annua* leaf and flower bud material by sonication in dichloromethane. The acid was partitioned into 0.1 N KOH, acidified with HCl, extracted into dichloromethane and then recovered by reverse phase HPLC. The purified acid was used to synthesize artemisinic alcohol, artemisinic aldehyde and amorpha-4,11-diene according to the method described previously [8] with the exception that 1.5 equivalent of diisobutyl lithium aluminum hydride was used to reduce the acid and the resulting alcohol/aldehyde mixture was subsequently resolved. The products were purified by reverse phase HPLC and confirmed by $^{1}$H NMR and EI$^{+}$-GC/MS [6,8].

GC/MS analysis was performed using an Agilent 6890 GC equipped with an auto-injector split 30:1 onto a DB-5ms column (30 m × 0.25 mm i.d., J&W Scientific) which was temperature programmed from 125 to 300 °C at 5 °C/min. The column was connected to a mass selective detector (Agilent 5973) operating under standard EI$^{+}$ conditions (70 eV).

### 2.2. Plant materials

*Artemisia annua* L. (seed source: Elixir Farm Botanicals, Brixey, MO, USA) were grown with 16 h/25 °C days and 8 h/20 °C nights to a height of approximately 1.2 m (about 3 months) then transferred to 12 h/25 °C days and 12 h/20 °C nights.

*Corresponding author. Fax: +1 306 975 4839.
*E-mail address:* Patrick.Covello@nrc-cnrc.gc.ca (P.S. Covello).

*Abbreviations:* AAFB, full-length flower bud cDNA library; AAGST, full-length glandular trichome cDNA library; CYP, cytochrome P450; DXP, 1-deoxy-D-xylulose-5-phosphate; EST, expressed sequence tag; GSTSUB, glandular-trichome-minus-flower-bud cDNA library

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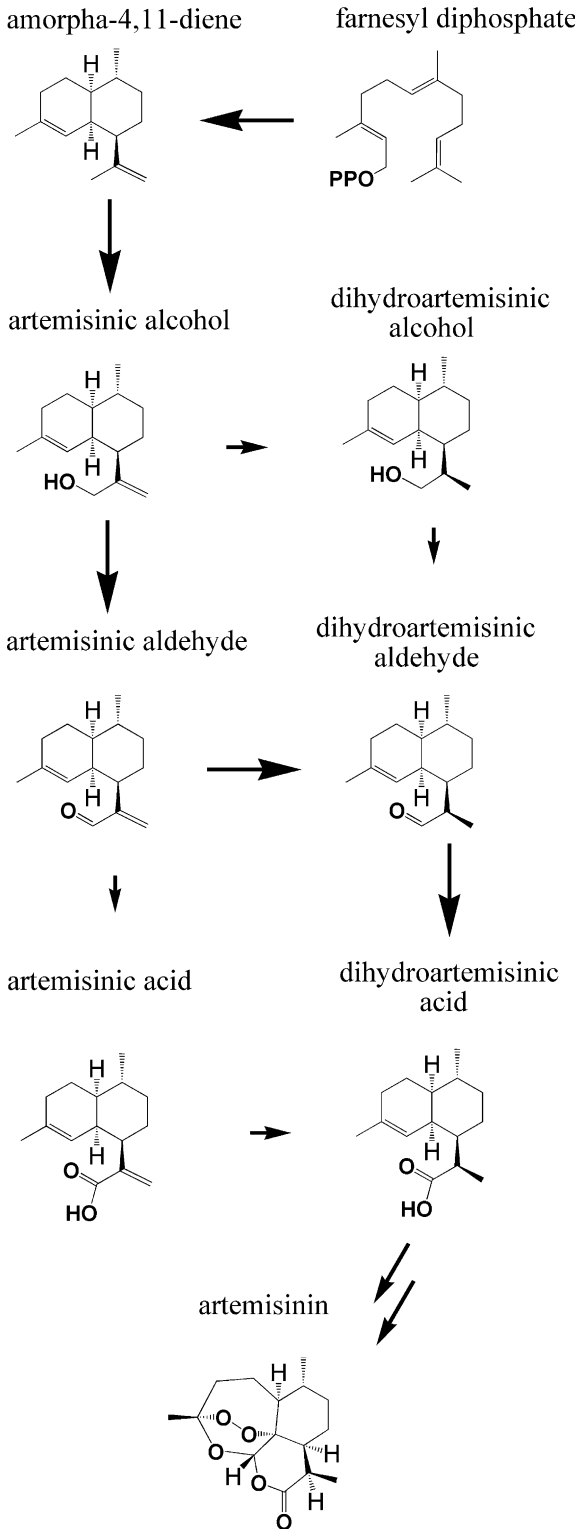


Fig. 1. Proposed pathway of artemisinin biosynthesis (redrawn from [6]).

Glandular trichomes were isolated from flower buds that developed after 19–21 12-h days as described previously with some modifications [9]. Flower buds were chilled in ice-cold water (1 h), then abraded using a cell disrupter (Bead Beater, Biospec Products, Bartlesville, OK, USA). The 350 ml chamber was filled with 20–30 g of plant material, 80–100 g of glass beads (0.5 mm diameter), XAD-4 resin (1 g/g plant material), and isolation buffer (25 mM MOPSO, pH 6.6, 200 mM sorbitol, 10 mM sucrose, 5 mM thiourea, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.6% (w/v) methylcellulose and 1% (w/v) polyvinylpyrrolidone ($M_r$ 40000)). The flower buds were abraded in an ice-chilled chamber by 3 pulses of operation of 1 min each separated by 1 min. Following abrasion, the contents of the chamber were filtered through 350 μm nylon mesh (Small Parts Inc., Miami Lake, FL, USA). The residual plant material and beads were scraped from the mesh and rinsed twice with rinse buffer (isolation buffer without polyvinylpyrrolidone and methylcellulose) that was also passed through the 350 μm mesh. The 350 μm filtrate was successively passed through a 105, 40 and 30 μm nylon mesh. Glandular trichomes were recovered from the 30 μm mesh.

### 2.3. cDNA libraries and ESTs

Total RNA used to construct the glandular trichome and flower bud EST libraries was extracted as described previously [10]. cDNA synthesis and construction of two full-length EST libraries (AAGST, trichome; AAFB, flower bud) were carried out with a Creator SMART cDNA Library Construction Kit (Clontech) using Long-Distance PCR following the manufacturer's instructions and using the vector pDNR-LIB. A subtracted cDNA library (GSTSUB) was constructed with a PCR-Select cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions using poly $A^+$ RNA isolated from glandular trichomes and flower buds (PolyATract mRNA Isolation System, Promega). Double stranded cDNAs of glandular trichomes and flower buds were used as tester and driver, respectively. Sequencing of randomly picked clones was performed on an AB13700 DNA sequencer using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and the M13 reverse primer. DNA sequence traces were interpreted, and vector and low quality sequences were eliminated using PHRED [11] and LUCY [12]. Clustering of the resulting EST dataset was done using STACKPACK [13] and sequence similarity was identified by BLAST [14].

### 2.4. Expression and characterization of CYP71AV1 in Yeast

The open reading frame (ORF) of a CYP designated CYP71AV1, encoded by the AAGST clone pKT101, was obtained through PCR using gene-specific primers 5′-CACCATGGCACTCTCACTGACCAC-3′ and 5′-CTAGAAACTTGGAACGAGTAACAAC-3′ and Vent polymerase (New England BioLabs, Cambridge, MA, USA). The resulting PCR product was cloned via the Gateway entry vector, pENTR/D-TOPO (Invitrogen) into a Gateway yeast expression vector, pYES-DEST52 (Invitrogen) to generate a yeast expression clone pKT011. The plasmid pKT011 was introduced into the *Saccharomyces cerevisiae* strain WAT11 [15] using S.c. EasyComp Transformation Kit (Invitrogen) to give the yeast strain WAT11/pKT011. Transformants were selected on synthetic complete medium lacking uracil (SC-U) containing 2% raffinose and grown at 30 °C for 24–48 h. Yeast cultures were initially grown in SC-U liquid medium containing 2% raffinose at 30 °C to an $OD_{600}$ of 2–3. Cells were centrifuged and resuspended in induction medium (SC-U containing 1% raffinose and 2% galactose) and grown at 30 °C for 12–16 h. Yeast microsomes were prepared according to Katavic et al. [16] and the microsomal pellet was resuspended in storage buffer containing 50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA and 20% (v/v) glycerol.

Microsomes of yeast cells transformed with pKT011 were assayed with amorpha-4,11-diene and other isoprenoid substrates, followed by GC/MS analysis. Enzyme reactions were initiated by adding substrate to a final concentration of 100 μM to 500 μl Tris–HCl buffer (25 mM, pH 8.0) containing 1 mM NADPH, 5 mM glucose 6-phosphate, 0.5 U of glucose 6-phosphate dehydrogenase, 300 μg of microsomal proteins, 2 mM DTT, 5 μM FAD, 5 μM FMN, 1 mM ascorbic acid and 10 μg/ml octadecane as an internal standard. Reactions were allowed to proceed for 30 min at 30 °C with shaking and immediately stopped by extracting twice with 500 μl diethyl ether. The ether extracts were pooled, dried down and dissolved in 20 μl of a 1:1 mixture of pyridine and *N*,*O*-bis(trimethylsilyl)acetamide (Sigma) followed by GC/MS analysis.

### 2.5. Analysis of CYP71AV1 expression in A. annua

For gene expression analysis total RNA was isolated from root tissue (RNeasy Plant RNA Isolation Kit; Qiagen), leaf and flower bud tissue (Trizol, Invitrogen), and isolated trichomes (guanidine chloride

[10]). First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) utilizing 200 ng of total RNA. The oligonucleotides 5′-GGACGATTTCGGAACTCATAA-3′ and 5′-CTCCTCTGGTAAAGCGCGTGTAG-3′ were used to amplify a 687 bp fragment of the CYP71AV1 transcript. Polymerase chain reactions were performed in 25 μl volumes using Advantage II DNA polymerase (BD Biosciences) according to manufacturer's instructions with 25 cycles and either 4 μl of cDNA and CYP71AV1 primers, or 1 μl of a 600-fold dilution of cDNA and 18S Universal Primers (Ambion). The specificity of the PCR primers used was confirmed by molecular cloning of the PCR products and the sequencing of 14 independent clones.

## 3. Results

### 3.1. Trichome isolation and cDNA library construction

In order to construct cDNA libraries enriched in clones representing glandular trichome-specific mRNAs, it was desirable to prepare relatively pure suspensions of glandular trichomes. This was accomplished by adapting previously described methods [9,17] for use with *A. annua* flower buds. At the flower bud stage, the accumulation of essential oil at the apex of trichomes is incomplete, suggesting that this was an appropriate stage to harvest trichomes active in isoprenoid biosynthesis. Trichome preparations were judged by microscopy to be approximately 90% glandular trichomes with the majority of the remaining material being fragments of T-shaped non-glandular trichomes.

Three cDNA libraries were constructed for use in gene identification relating to *A. annua* glandular trichomes. A total of 1928, 2812, and 1041 ESTs from the trichome-minus-flower-bud subtracted (GSTSUB), full-length trichome (AAGST), and full-length flower bud (AAFB) libraries, respectively, were subjected to similarity searches and clustering. Inspection of the ESTs and associated sequence similarities for the three libraries reveals their apparent value in identifying genes, which are expressed preferentially in the glandular trichomes. Not surprisingly, given the biosynthetic activity attributed to glandular trichomes, the two libraries derived from trichomes (GSTSUB and AAGST) are enriched in cDNAs corresponding to isoprenoid biosynthesis, relative to the AAFB library. Consistent with a major role for trichomes in monoterpene biosynthesis, all of the enzymes of the DXP (1-deoxy-D-xylulose-5-phosphate) pathway from DXP synthase to 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase [18] are represented in at least one of the GST libraries. Perhaps the most important indicator of sesquiterpene biosynthetic activity in the trichomes, from which the libraries were made, is the frequency of sequences corresponding to amorpha-4,11-diene synthase. This gene represents 3.9% and 0.9% in the GSTSUB and AAGST collections, respectively, but it was not represented in the AAFB collection.

### 3.2. CYP71AV1 sequence and expression analysis

In order to investigate the role of CYP-dependent enzymes in trichome metabolism and particularly in amorpha-4,11-diene oxidation, ESTs corresponding to CYPs were examined. A range of CYP subfamilies were represented at the level of 0.3%, 1.2% and 0.1% in the GSTSUB, AAGST and AAFB collections, respectively. This included a cluster, represented by 1 GSTSUB clone and 11 AAGST clones, with sequences similar to CYP71D subfamily members. By virtue of its similarity to terpene hydroxylases, this cluster, deemed to represent a single gene, was chosen for further study. By additional DNA sequencing and sequence analysis, the clone pKT101 was determined to include a full-length ORF matching the consensus sequence for the 12 highly similar cDNA clones in the cluster. The nucleotide sequence of pKT101 was submitted to the Genbank database (No. DQ315671). The corresponding 488 amino acid polypeptide included the sequence FGAGRRMCPG (residues 355–364), which matches the consensus for the CYP heme-binding domain. The sequence was designated CYP71AV1, a new CYP71 subfamily, and shows the highest degree of amino acid sequence similarity to CYP71D4 (51% identity; Genbank Accession No. CAC24711), an enzyme of unknown function from *Solanum tuberosum*. In terms of enzymes of known function, it is most similar to CYP71D16 (49% identity), or cembra-2,7,11-triene-4-ol monooxygenase, from *Nicotiana tabacum* [19].

### 3.3. CYP71AV1 is a trichome-specific sesquiterpene monooxygenase

For functional studies, microsomes from yeast expressing CYP71AV1 were assayed with amorpha-4,11-diene followed by analysis by GC/MS after trimethylsilylation. Fig. 2 shows the results of this analysis indicating the CYP71AV1-dependent formation of a new GC/MS-detectable compound with a retention time of 16.6 min. The retention time and mass

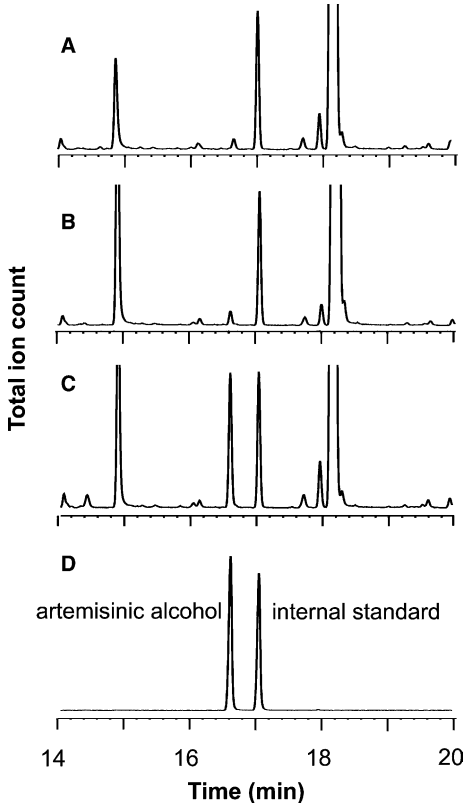


Fig. 2. In vitro hydroxylation of amorpha-4,11-diene by CYP71AV1. GC/MS traces of extracts of enzyme assays containing the amorpha-4,11-diene and microsomal fractions isolated from (A), the control yeast strain WAT11/pYES-DEST52; (B) yeast strain WAT11/pKT011 without added NADPH; (C) yeast strain WAT11/pKT011 (complete assay); and (D) chemical standards.

spectrum of this compound was identical to trimethylsilylated artemisinic alcohol (Fig. 3). Only traces of the compound were found in assays in which NADPH was excluded and it was not detected in assays with WAT11/pYES-DEST52 control microsomes. The mass spectrum of the small peak near 16.6 min in Fig. 2A bore no resemblance to that of trimethylsilylated artemisinic alcohol (data not shown). Thus, CYP71AV1 appeared to act as an amorpha-4,11-diene 12-monooxygenase.

In some complete assays using CYP71AV1-containing microsomes, a minor product with a retention time of 14.4 min and a mass spectrum matching artemisinic aldehyde [6] was observed (see Fig. 2C and data not shown). This was investigated further by testing both artemisinic alcohol and artemisinic aldehyde as substrates. CYP71AV1-containing microsomes were found to be active on both compounds, as indicated in Fig. 4. GC/MS analysis showed that the main products of the alcohol and aldehyde substrates were artemisinic aldehyde and artemisinic acid, respectively. Thus, based on the in vitro results presented, CYP71AV1 is multifunctional and capable, in three steps, of oxidizing amorpha-4,11-diene to artemisinic acid, via alcohol and aldehyde intermediates.

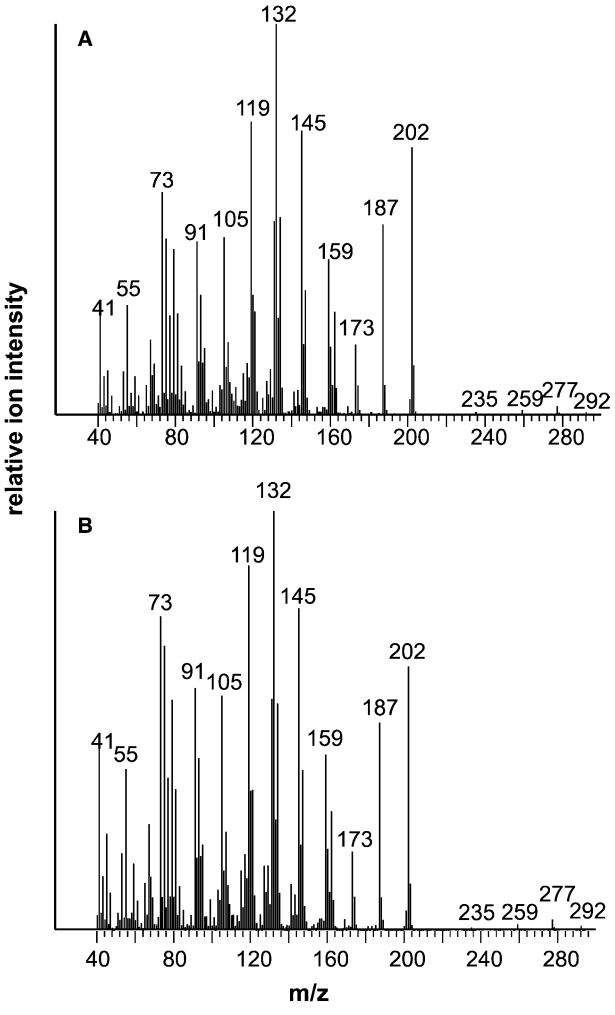


Fig. 3. Mass spectra of the trimethylsilyl derivatives of the product of the complete enzyme reaction containing WAT11/pKT011 microsomes (A) and synthetic artemisinic alcohol (B).

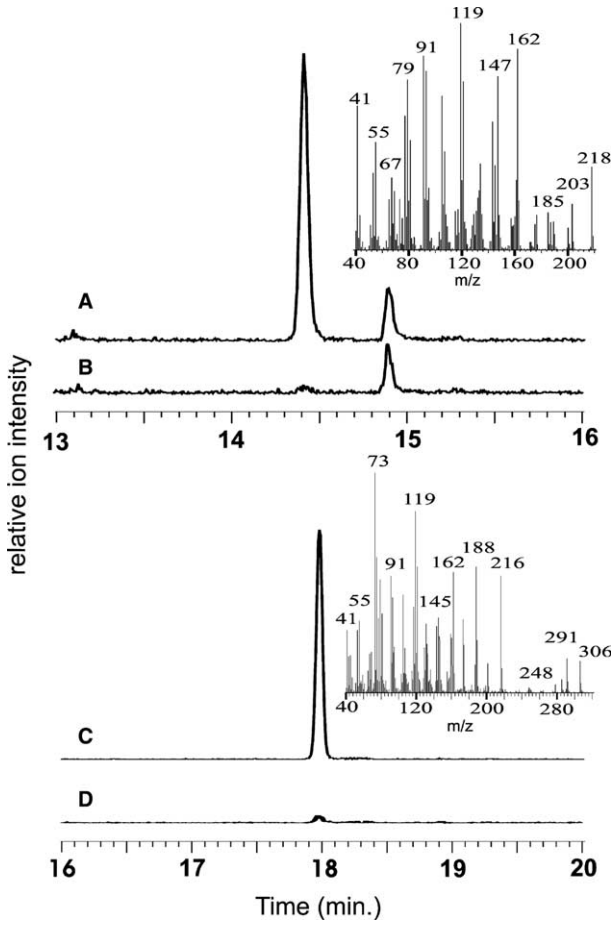


Fig. 4. Oxidation of artemisinic alcohol and artemisinic aldehyde by CYP71AV1. GC/MS traces of trimethylsilylated extracts of enzyme assays containing artemisinic alcohol (A and B, monitored at $m/z$ = 218) and artemisinic aldehyde (C and D, monitored at $m/z$ = 306) and microsomal fractions isolated from the yeast strain WAT11/pKT011 (A and C) and the control yeast strain WAT11/pYES-DEST52 (B and D). Mass spectra of compounds identified as artemisinic aldehyde (trace A, R.T. = 14.43 min) and trimethylsilyl artemisinate (trace C, R.T. = 18.05 min) are shown.

To investigate the specificity of CYP71AV1, a variety of other compounds were tested as possible substrates, including the monoterpenes, limonene, α-pinene, β-pinene and pinocarveol; and the sesquiterpenes (−)-alloisolongifolene, caryophyllene, (−)-α-gurjunene, (+)-γ-gurjunene, (+)-ledene, (+)-β-selinene and (+)-valencene. CYP71AV1-containing microsomes were not found to be active on any of the above compounds. This was confirmed for (+)-β-selinene, which has an isopropenyl moiety similar to amorpha-4,11-diene, by performing an assay with both (+)-β-selinene and amorpha-4,11-diene. The specificity of CYP71AV1 for amopha-4,11-diene was confirmed by the formation of artemisinic alcohol without the detection of any hydroxylation products of (+)-β-selinene (data not shown).

Expression analysis of CYP71AV1 in *A. annua* tissues indicates that it is most highly expressed in trichomes (see Fig. 5). The moderate expression observed for flower buds presumably reflects their high density of trichomes. Low level RT-PCR products could be observed for leaves and roots (data not shown).

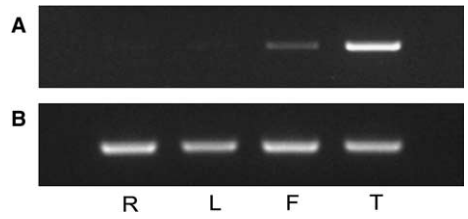


Fig. 5. CYP71AV1 is highly expressed in glandular trichomes. Ethidium bromide-stained agarose gels showing RT-PCR results for both CYP71AV1 (A) and 18S rRNA (B) for root (R), leaf (L), flower bud (F) and glandular trichome (T).

## 4. Discussion

Despite the widespread occurrence and range of important bioactivities attributed to sesquiterpene lactones, relatively little is known about their biosynthesis and the genes that control it [20]. Furthermore, little is known about the biosynthetic, transport, regulatory and developmental processes occurring in glandular secretory trichomes which populate the surfaces of a large number of plants and are so important in ecological interactions [21]. The specialized EST collection described in this paper is an important tool for the elucidation of various trichome-specific processes. In particular, we have taken advantage of it, in combination with heterologous expression, to characterize amorpha-4,11-diene monooxygenase (CYP71AV1), a CYP which catalyzes an important regioselective oxidation in artemisinin biosynthesis. The enzyme has the additional ability to oxidize the alcohol, to the corresponding aldehyde and acid. Similar activity has been observed by other CYPs [22]. Further work is required to study the complete catalytic properties of the enzyme, including its role in the oxidation of artemisinic alcohol.

The role of CYP71AV1 in the hydroxylation of amorpha-4,11-diene is clearly important in artemisinin biosynthesis. The subsequent route to artemisinin is less clear. Most evidence implicates dihydroartemisinic acid as a late precursor to artemisinin which is derived from artemisinic alcohol by oxidation at C-12 and reduction of the C11–C13 double bond (Fig. 1). This is based on in vitro biochemical evidence [6], as well as the conversion of dihydroartemisinic acid to artemisinin both in vivo [23], and in vitro, in an oxygen-dependent non-enzymatic fashion [24]. Although Bouwmeester and coworkers [6] have suggested that soluble enzymes are involved in C-12 oxidation, CYP71AV1 presents an alternate membrane-bound oxidative activity which may be important in conversion of artemisinic alcohol and/or later intermediates.

The characterization of CYP71AV1 has implications for understanding sesquiterpene lactone biosynthesis in general. It is notable that enzyme activity similar to that reported for CYP71AV1 has been reported in chicory (*Cichorium intybus*) extracts [25]. The sesquiterpene lactones of chicory are derived from germacrene A and both germacrene A and amorpha-4,11-diene are hydroxylated by what appears to be CYP activity in chicory root extracts [25]. This, and the data presented in this paper, suggests that the biosynthesis of a range of sesquiterpene lactones in various members of the Asteraceae may include oxidation of sesquiterpene precursors catalyzed by CYP71AV1 homologues.

The cloning and characterization of CYP71AV1 also has important implications for biotechnology and the supply of antimalarial drugs. There is currently a shortage of artemisinin for the semi-synthesis of such drugs [26]. As well, the economical long-term supply of artemisinin from *A. annua* is limited by its low levels in the plant. The cloning of CYP71AV1 provides an opportunity to improve the supply of artemisinin through genetic engineering of plants or microorganisms, either through semi-synthesis or in combination with additional genes.

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